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THE DIFFERENTIATION OF BACTERIA OF THE COLON-AEROGENES FAMILY[1]

By William Mansfield Clark

When the author was asked to present a paper before this meeting he gladly accepted for he felt that some of the results which Mr. Rogers and himself had obtained in various studies of the so-called colon group of bacteria may be of some use not only in future studies of water supplies but in rapidly and accurately meeting some of the problems which will arise in connection with the water supplies of our camps and our armies in the field. At the same time the author is painfully aware that he is not familiar with the details or the special problems of water bacteriology and he, therefore, begs indulgence while he reviews some studies in which he has been particularly interested.

For a long time a distinction has been made between B. coli and B. aerogenes, but in the heterogenous collection of organisms which were fished from feces, water, milk, etc., it was not always easy to make a clear distinction by the tests at hand. The reason is perfectly plain.

If we study not only the more modern work but especially the researches of Harden it is evident that the products of fermentation by B. coli on the one hand and B. aerogenes on the other are very similar and differ chiefly in the quantitative proportions.

The quantitative differences in gas production were recognized by Theobold Smith and upon these differences, Dr. Smith established the use of his fermentation tube. Because the characteristics studied differ only in a quantitative way, while the method involves sources of serious analytical error, the Smith fermentation tube has been of very limited usefulness. Harden, Harden and Walpole, and Thompson established somewhat more accurately the gas ratios but it remained for Keyes and Keyes and Gillespie not only to furnish reliable data for B. coli but to frame the method of gas isolation which has proved so useful in the more extensive investigations by Rogers and his collaborators.

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[1] Read before the Chemical and Bacteriological Section at the Richmond Convention, May 10, 1917.

When bacteria of the colon-aerogenes family are grown in bulbs evacuated by a mercury pump and the CO_2 and H_2 liberated in the anaerobic fermentation of dextrose are collected with the aid of such a pump and analyzed accurately the ratio CO_2/H_2 has been found to be a most valuable means of differentiation. We have now the analyses for about 660 cultures.

In their study of cultures isolated from milk and milk products, Rogers, Clark and Davis found that approximately half gave a low gas ratio $CO_2 / H_2 = 1.06$. The others gave decidedly higher and more variable ratios. Taking up a search for possible sources of these two groups, Rogers, Clark and Evans found that of 150 cultures isolated from bovine feces all but one were of the low ratio type. Where, then, are the high ratio organisms to be found? All possible sources are of course not known, but in a study of 166 cultures isolated from grain, it was found that 150 were of the high ratio type. Recently it has been found that high-gas-ratio cultures may be isolated from both bovine and human feces by special methods, but that the predominant type found in both human and bovine feces are of the low-ratio type.

The potential sanitary significance of such results is perfectly obvious. It will perhaps seem more pertinent if we recall that Rogers, following the course of several streams, found the low-ratio colon cultures increasing in relative numbers with the entrance of sewage and decreasing in relative numbers as the streams purified themselves and passed on into cleaner surroundings.

The gas analysis methods employed are necessarily laborious and time consuming and therefore not appropriate for routine work. But fortunately a test has been developed which correlates with the gas ratios. The history of the development of this test would lead into some technical details which can be appreciated only by a careful study of the published data, but the principles involved may be outlined as follows:

In its fermentation of dextrose, B. coli does not cease its activity with the production of any definite quantity of acid but only when the actual ionized acid, as measured by the dissociated hydrogen ions reaches a certain concentration. This value may be measured electrometrically by means of the hydrogen electrode, but for all ordinary purposes it is sufficient to measure it with an indicator.

The proper indicator to use in this instance is methyl red, which, if added to a culture of B. coli in definite quantity, will always assume about the same red color. It is evident that, if this lethal or limiting hydrogen ion concentration is to be reached, the culture must be furnished with sufficient sugar so that the acid formed therefrom may neutralize those substances in the medium which tend to combine with and suppress the dissociation of the acid. These substances are technically known as buffers. As the buffer action of the medium is increased, increasing amounts of the acid-forming sugar must be supplied. Now let the sugar content be adjusted to the buffer action so that B. coli can just reach its limiting hydrogen ion concentration. Under these circumstances, what will an aerogenes culture do? For every molecule of sugar that an aerogenes culture decomposes it forms very much less acid than a coli culture. Therefore, under the circumstances, an aerogenes culture can not overcome the buffer action of the medium. It can not reach that limiting hydrogen ion concentration characteristic of B. coli, its other activities are not suppressed, it continues to decompose what little acid has been formed, after a day or two it begins to make the medium more alkaline and the gross result is that the hydrogen ion concentration is very much lower than that of a colon culture. It only remains to make this difference evident to the eye. Since methyl red turns red in the one case and yellow in the other it is a suitable indicator for the purpose. Hence the test has come to be known as the methyl red test.

These are the principles involved. To apply them it was necessary to devise a medium in which the buffer action and dextrose content were properly adjusted. Such a medium is composed of 0.5 per cent K_2HPO_4, 0.5 per cent dextrose and 0.5 per cent Witte peptone.

This medium is inoculated with pure cultures of the organisms to be tested and grown five days at 30°C. To test the resulting hydrogen ion concentration it is necessary only to add some methyl red. Coli cultures are red to methyl red, aerogenes cultures yellow. This test was found to correlate perfectly with the gas ratios when typical organisms were used.

One of the essential components of this medium is Witte peptone. The war has made the price of this preparation prohibitive and therefore several workers have tried to substitute other peptones. Now there is no particular virtue in Witte peptone which makes it necessary to use it in this instance, but it must be clearly emphasized that it takes part in the buffer action of the medium, upon the proper adjustment of which the very essence of the test is based. When, therefore, other peptones are substituted without any regard to the principle mentioned, uncertain results can safely be predicted. It has been a matter of great surprise to the author that several bacteriologists, without any regard for the very simple and essential principles of the method, have indiscriminately changed the composition of the medium. Apparently the question of economy or the mere curiosity to see what would happen had greater weight than the adherence to a scientific principle.

There is no apparent reason why peptone preparations other than Witte's can not be substituted provided the relation of the resulting buffer action to the sugar content is properly adjusted. But rather than work over this question again, Dr. Lubs and the author decided to devise a synthetic medium. The principles applied were the same. The proper buffer action was provided by the salts of phosphoric and phthalic acids and the nitrogen by aspartic acid. The relative proportions of these were so adjusted that they furnished not only the proper buffer action but also the proper initial hydrogen ion concentration, for it is now known that adjustment of the reaction of a medium by the old titration method has nothing but an empirical significance for particular media and that the only valid general method of adjustment is in terms of hydrogen ion concentration. Then, by trial, the proper dextrose content was fixed. The resulting medium is composed of 0.7 per cent anhydrous Na_2HPO_4, or 0.88 per cent of the salt which contains two molecules of water, 0.2 per cent KH phthalate, 0.1 per cent aspartic acid and 0.4 per cent dextrose.

Tests of 229 typical cultures of the colon aerogenes family have been made on this medium. The correlation between the differentiations so made and those made with the gas ratios was 100 per cent perfect.

The author has mentioned so far the tests made upon what have been called typical cultures. By typical cultures in this instance are meant those organisms whose gas ratios and gas volumes, when accurately determined, conform to one or the other of the two types of values which the study of some 660 cultures has clearly established as characteristic. From time to time there have appeared in the collections of the Bureau of Animal Industry or there have been sent to the Bureau by others, cultures which in one way or another did not conform to either type. The methyl red test when applied to these cultures is more or less uncertain.

It is of course impossible to say how frequently or under what circumstances similar organisms will occur in the collections gathered in the routine examination of diverse waters. The Bureau's results indicate that they are comparatively rare and not likely to interfere seriously with sharp differentiations.

If these atypical cultures, which can not be definitely classified by any test known to the author, are excluded, there is a total of 374 cases in which the correlation between the methyl red test and the accurately determined gas ratio is perfect. As was pointed out in an earlier paper, a similar degree of correlation between any two tests used in the colon-aerogenes group was unknown.

Recently Levine in studying his collection of coli-aerogenes cultures discovered an almost perfect correlation between the differentiations made with the Voges-Proskauer reaction and those made with the methyl red test. By means of this correlation Levine was able to show that some of the conclusions regarding the distribution of these bacteria which had been reached in older studies with the Voges-Proskauer reaction were confirmed by the more careful work of Rogers and his collaborators.

According to Harden, "Voges and Proskauer's reaction . . . . appears to be due to acetyl methyl carbinol, which is formed by the action of the bacteria on the glucose of the medium. In the presence of potash and the air this is oxidized to diacetyl which then reacts with some constituent of the peptone water."

Since the revised medium described contains no peptone or any protein body, except such as may be formed by the synthetic action of the bacteria, it is necessary to supply such a substance if the Voges-Proskauer reaction in this new medium is to be observed. Harden and Norris have shown that various proteins and peptones react with diacetyl in the presence of alkali to give the eosin-like fluorescent color characteristic of this test. Since casein may easily be obtained in a pure state and reacts with diacetyl, it is a suitable reagent for this present purpose. It is added after the growth of the cultures together with the strong alkali and produces in the colorless synthetic medium an intensity of color which far exceeds that of any Voges-Proskauer reaction the author has ever seen in other media.

Colon cultures with low gas ratio do not give the reaction. High-gas-ratio cultures generally do.

For reasons which will be presented in detail in a forthcoming paper, the author does not place the reliance on this test that he does on the methyl red test. The chief reason is that in certain cultures the reaction is faint and fleeting, and may easily be missed. By means of very careful observations, however, for all the typical cultures tested a perfect correlation with the Voges-Proskauer test and the gas ratios has been established. This, then, furnishes three tests which correlate perfectly.

Correlations between the gas ratio and the Voges-Proskauer test have been established by Harden, Walpole and Thompson. Correlations between the methyl red test and the Voges-Proskauer test have been established by Winslow and Kligler, Levine, Hulton, Greenfield and Johnson. Dr. McCrady, of Montreal, has recently reported results which are in accord with the work mentioned.

Lastly, the Bureau of Animal Industry has established in its own work a correlation between the gas ratios, the Voges-Proskauer tests and the methyl red tests which leaves no doubt that the methyl red test is a simple, practical and reliable method for differentiating the two chief groups of the colon-aerogenes family.

Perhaps the author should here leave the subject for he has personally been concerned only with the development of the method and the technique of its use and not with interpretations of the sanitary significance of the results. But he desires to emphasize strongly a matter which, it seems to him, should be kept perfectly clear in any discussion of the merits of the test.

If it is desired to differentiate between the high and the low gas ratio cultures, between those two types which may be conveniently called coli and aerogenes, a reliable method will be found in the methyl red test. Of this there is no doubt. But the problems which water works managers face are more complicated. They are called upon not only to determine the flora of the waters placed under their care but also to interpret the sanitary significance of the flora that are found.

It is certainly suggestive to know that in the feces of man and domestic animals the methyl red positive type of the colon aerogenes bacteria is dominant, and in grain and certain soils the methyl red negative type predominates. But it is well to remember that a careful search will reveal B. aerogenes in feces and B. coli in virgin soil and in waters situated far from the habitations of man and domestic animals.

It is evident, therefore, that the significant thing to look for is the relative numbers of each type in the material under examination, as Rogers did in his water work. In the extension of such studies, there will be involved several important questions, such as the relative rates of growth or destruction of each type when it enters waters of different chemical composition and of different physical conditions from various sources of pollution, and also the examination of yet unstudied potential sources of each type.

Perhaps such questions will have to be carefully and extensively investigated before one can draw very certain conclusions regarding the sanitary significance of the data furnished by the methyl red test, but it seems to the author that in the mean time the test should be included in the routine examination of water supplies for the following reasons: In the first place, it is as simple a test as any in use and at the same time is based upon phenomena the gross aspects of which are now known far better than are those of several other tests which might be mentioned. In the next place it correlates with other tests to a degree unknown among the tests formerly employed. Finally, laying aside any opinion that it may be used as a direct index of pollution, it does differentiate two very distinct types of bacteria which have frequently been confused in the routine tests of the past. It therefore gives the water works superintendent a better acquaintance with the flora of the supplies placed in his charge. Those who guard these trusts in more than a perfunctory manner probably depend upon such an intimate acquaintance to a greater degree than can be expressed in the formal terms of standard methods or court room pronouncements.

The author's belief is that the methyl red test will furnish the basis for some good guesses. It will at least be a valuable instrument for some practical researches.

DISCUSSION

F. W. Green: This subject of the hydrogen ions content, the neutrality of the media and the relationship between the two, appears to the speaker one of colloids. The condition of the media, as to whether these colloids are suspensoids or emulsoids, is a very important factor in the reaction, and, therefore, it is necessary to take into account the electrical potential of the media. The speaker knows of no method of determining this and thinks that both in laboratory work and also in the reactions of the general supply this is a most important factor and should be taken into account. Tests should be devised whereby it will be possible to determine the amounts of colloids that are present and whether they are suspensoids or emulsoids.

JOSEPH RACE: Everyone who has had experience in water bacteriology must have a feeling of gratitude toward the workers at Washington for the able and painstaking efforts which they have made to elucidate the whole question of B. coli. The work which they have performed in the last two or three years has advanced our knowledge of B. coli more than all that has taken place during the last decade. The author has mentioned several important points and has discussed the technique in considerable detail. One point that he emphasized was the importance of the delicate adjustment of the buffer to the sugar, and to water works men it is important to know if the usual medium has this adjustment. The speaker believes it has not. The usual practice in the determination of type is to plate out from the liquid enrichment medium on the first signs of gas. That raises the question of the relative rate of reproduction of the various types in a given medium. Taking, for example, a fifty-fifty mixture of the fecal and grain types in lactose broth or bile; what will be the relative proportions after twenty-four and forty-eight hours? There is no guarantee that the colonies fished in the usual way are not all descendants from one common organism and the only accurate method for the determination of type is to plate out the original sample on a solid medium. On looking over his results for the last six to eight months it appears to the speaker that if gas is present in the fermentation after twenty-four hours the majority of the organisms are of the fecal type: after forty-eight incubation the majority are of the grain or negative methyl red type. This seems to indicate that the grain type is more resistant than the fecal type under the conditions obtaining in the fermentation tubes.

WILLIAM MANSFIELD CLARK: One of the accepted points in which colloids differ from crystalloids is the size of the particle. This carries with it certain accentuations of properties which are held in common by both classes. Now it is true that in culture media we frequently have matter in the colloidal state but the essential point at issue is that these colloids act as neutralizers in essentially the same way as more familiar acids and bases. The neutralizing action of casein is an example. There are, of course, substances which differ from this in that the absorption of acids and bases is a phenomenon of surface condensation. Charcoal is not regarded as an acid or a base, and yet upon charcoal it is possible to have condensation of an acid and the resultant effect is as if the acid were neutralized. From the practical point of view the author thinks these particular explanations may be disregarded because there are methods which determine with reasonable accuracy, and simply, the actual hydrogen ion concentration of a medium; and so far as the author knows it is this value as determined by these methods which is found to be significant for the growth and metabolism of bacteria.

It would be very nice to be able to distinguish between the neutralizing effect of colloids as colloids and all other bodies which are acting by virtue only of their acidic or basic properties, but for most practical purposes it is at present unnecessary to make such a distinction in adjusting and testing culture media.

To determine by the simple methods at hand the hydrogen ion concentration which you actually have, whatever the conditions causing it, and to adjust media in accordance with the particular value which is found to be advantageous for the particular organism you are studying, is the first and necessary step.

The subject of enrichment media is one which we have not investigated, but there are involved certain theoretical considerations which it seems to the author ought to be recognized very clearly. B. coli has an optimum rate of growth slightly above the true neutral point, a hydrogen ion concentration represented by P_h 6.8 perhaps. On either side of that point the rate of growth will not decline rapidly at first, but regions may soon be reached where the rate will decline exceedingly rapidly. The relative rates of growth for B. aerogenes at different hydrogen ion concentrations has, so far as the author knows, not been studied and therefore he does not think it is possible to reach any definite conclusion regarding the relative increases in the number of each type when growing in enrichment media. Of course the question may have been attacked from an empirical point of view, with the medium strictly limited to one composition and one reaction. No very useful generalization can be reached in that way.

In the study of all such problems it is exceedingly important, vitally important, to control the true reaction of the medium. This subject has been studied extensively by Dr. Lubs and the author, who have shown that the colorimetric method of measuring the hydrogen ion concentration of culture media can be used by any bacteriologist with ordinary chemical training. The author thinks the method will have to be applied before we know definitely where we stand in regard to several aspects of enrichment media.

In this connection the author desires to mention one incident. A certain official medium used for testing disinfectants contained a rich buffer in its content of beef infusion. It had been empirically established that this medium should have a certain "reaction" which, the author believes, was plus 1.5. Some one, the author does not know the history of the case and so may not state it with historical accuracy, some one thought it would be well to "simplify" the medium by substituting for the beef infusion 0.3 per cent Liebig's Extract. This was done, but the formula was left with the same degree of titratable acidity which had worked all right in the old medium. There was considerable wonder when it was found that the new medium would not work, but it was soon found why it would not work, for Doctor Phelps discovered that the new medium had a hydrogen ion concentration near the optimum acid agglutination point of B. typhi. By applying the rational method of adjustment they are now getting good results with the new medium.

The author might go on indefinitely with the citation of other cases. For instance, the product of one of our American manufacturers was seriously threatened because people insisted upon applying to media constructed with this peptone the old standards of reaction which had been empirically established for Witte peptone. Now that adjustments are made in terms of hydrogen ion concentration, as good and sometimes better results can be obtained with the American product.

In what degree the true reaction of an enrichment medium will influence the relative numbers of different bacteria in mixed cultures the author can not say, but he is sure little confidence should be placed in the process until this factor is experimentallv known.